Developing Tools to Monitor *Vibrio cholerae* Type VI Secretion During Range Expansion on Solid Surfaces

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During Range Expansion on Solid Surfaces

by

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Developing Tools to Monitor *Vibrio cholerae* Type VI Secretion During Range Expansion on Solid Surfaces

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AmpR	Ampicillin Resistance Gene
CFP	Cyan Florescence Protein
ColE1	Origin of Replication
Dap	Diaminopimelic Acid
Dap^-	Diaminopimelic Acid Mutant
GFP	Green Fluorescent Protein
HGT	Horizontal Gene Transfer
LB	Luria broth
OFP	Orange Florescence Protein
oriT	Origin of Transfer
PCR	Polymerase Chain Reaction
pUC18	High Copy Plasmid
RFP	Red Fluorescent Protein
T6SS	Type VI Secretion System
$T6SS^+$	Constitutively Expressing T6SS
$T6SS^-$	No Functional T6SS
WT	Wild type

SUMMARY

The waterborne bacterium *Vibrio cholerae* causes the fatal cholera diarrhea, and thrives in aquatic environments attached to chitinous surfaces with other bacteria. *V. cholerae* has been used to study natural competence, which promotes DNA uptake through horizontal gene transfer (HGT). A newly described Type VI Secretion System (T6SS) that *V. cholerae* employ to kill neighboring cells could increase the chance for a competent *V. cholerae* to take up and utilize the released DNA. The T6SS apparatus, which is similar to a bacteriophage spike, injects toxic effector proteins into prey cells causing lysis that can aid *V. cholerae* in acquiring DNA from its neighbors. Although previous studies have investigated the regulation and mechanism of T6SS in *V. cholerae* at a single cell level, the role of T6SS in the dynamics of mixed bacterial populations, such as those found in the environment, remains poorly understood. Fluorescence microscopy was used in this study to determine the effect of T6SS by growing *V. cholerae* predators and isogenic prey populations on agar surfaces as a model for competitors undergoing spatial expansion. These conditions may mimic conditions encountered on chitinous surfaces in marine settings. In this study, plasmids expressing green and red fluorescent proteins were constructed to visualize the predator and prey populations. Preliminary spatial expansion experiments were ultimately performed using different fluorescent protein alleles that were encoded on the chromosome of competing bacteria. Preliminary results suggest that the presence of a functional T6SS plays an important role in competition. The tools developed for this study are now being used to

study interaction between diverse *V. cholerae* isolates, and as a platform for experimental evolution.

CHAPTER 1

INTRODUCTION

Many bacteria utilize protein secretion systems to transport proteins outside of the cell. These translocated proteins can play an important role in competition with other bacteria and also in interaction with hosts that are critical to cause diseases. There are more than six types of secretion systems, and the Type VI Secretion System (T6SS) was discovered in 2006 (Cascales and Cambillau, 2012).The T6SS resembles a phage-like apparatus, that can puncture adjacent cells and inject a variety of toxic effector proteins, in a manner similar to the delivery of viral genetic material into its host cells (Cascales and Cambillau, 2012). There is a great deal of interest in understanding the contributions that this apparatus makes to bacteria that utilize them.

The waterborne human pathogen, *Vibrio cholerae*, serves as a model organism to study bacterial behaviors, such as biofilm formation, quorum sensing, and pathogenesis (Ng and Bassler, 2009). Previous studies showed *V. cholerae* uses the T6SS to deliver toxic effector proteins to a variety of cell types, including human macrophages, protozoan predators (*Dictyostelium discoideum*), and bacterial competitors (*Escherichia coli*), causing cell death (Bingle *et al*., 2008; Cascales and Cambillau, 2012; MacIntyre *et al*., 2010). In *V. cholerae*, there are three chromosomal T6SS loci, including a large cluster and two auxiliary clusters (Fig. 1). The large cluster includes the major structural genes for T6SS apparatus assembly, such as *vasK*. Two auxiliary clusters also have essential genes like *hcp* that are required for functional T6SS. Each cluster has a pair of genes

encodes an effector and adjacent immunity factor (Fig. 1), which the immunity factor can neutralize the specific toxic effectors.

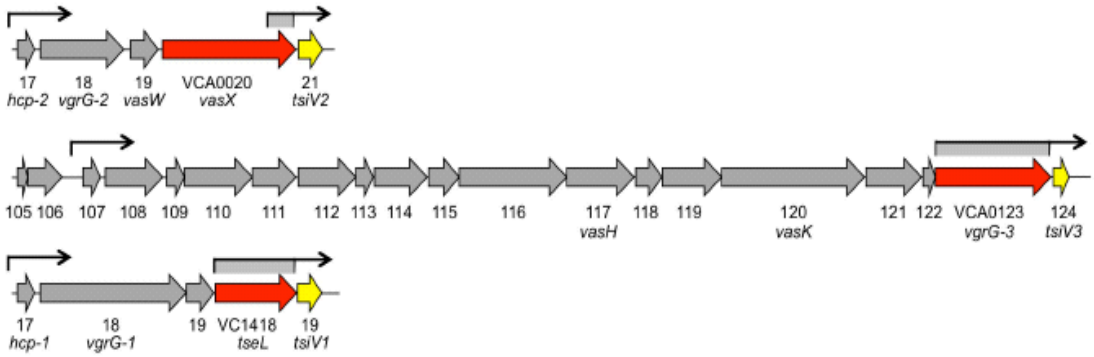

Figure 1. T6SS chromosomal loci in *V. cholerae*. Middle: The large cluster that is composed of the T6SS structural genes, such as *vasK*, and a pair of effector and immunity gene highlighted in red and yellow, respectively. Top and Bottom: Two auxiliary clusters with effector (red) and immunity (yellow) gene pairs.

To cause disease, *V. cholerae* colonizes the intestine and secretes the cholera toxin responsible for the fatal cholera diarrhea, and some evidences support that T6SS may have a role in the pathogenesis of humans caused by cholerae toxin (Colwell, 1977; Ma and Mekalanos, 2010). However, *V. cholerae* also thrives in aquatic environments, often attached to chitinous material (zooplankton molts, crab shells) in biofilms with other competing bacteria (Huq *et al.*, 1983). The effects of T6SS-mediated killing on mixed populations of bacteria has not yet been studied and requires genetic tools and fluorescence microscopy methods described below that are the focus of this research project.

On chitinous surfaces (i.e: crab shell), *V. cholerae* express genes for a competence apparatus to take up extracellular DNA from the environment. When of sufficient sequence identity, the DNA that is acquired can be incorporated into the chromosome and provide the bacterium with a novel ability, such as antibiotic resistance, which can

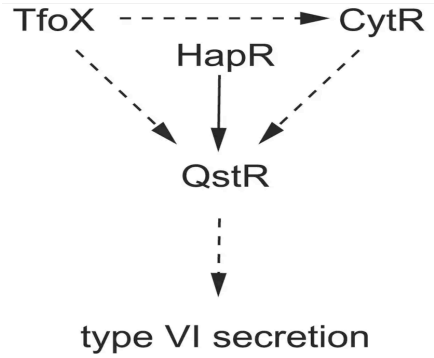

Figure. 2. The Model of TfoX, CytR, HapR, and QstR in T6SS. T6SS requires induction from the four regulators as shown, but the overexpression of QstR bypasses the need for TfoX, CytR, and HapR (Watve, Thomas & Hammer, 2015.)

increase fitness of the cell (Sun, 2013). This process is called natural transformation. In the *V. cholerae* clinical reference isolate C6706, the T6SS apparatus and the competence apparatus are regulated by four transcription factors: TfoX, CytR, HapR, and QstR (Fig. 2.; Sun, 2013). Interestingly, this regulatory pathway also controls expression of the T6SS. Because competence and T6SS are coordinately expressed, Borgeaud *et al*. hypothesized that when *V. cholerae* grows on chitin, T6SS is induced, causing the lysis of neighboring prey cell that releases internal components, including DNA. At the same time, the competence genes are activated that allow *V. cholerae* to take up DNA through HGT (Borgeaud *et al.*, 2015). Based on this model, it is predicted that T6SS provides an advantage that allows *V. cholerae* to compete with neighboring cells and colonize surfaces.

Although the function of T6SS has been well documented, the mechanism by which the T6SS is regulated in bacteria remains less clear. For example, the T6SS in some strains of *V. cholerae* (V52) is constitutively expressed, whereas the clinical reference strain C6706, a common model strain, studied in our lab and the others, is induced when the *tfoX* regulatory gene is expressed in the presence of chitin (Fig. 2.; Borgeaud *et al.,* 2015; Watve, Thomas & Hammer, 2015). We have engineered a T6SS^{+} "max killer" of *V. cholerae* C6706 to constitutively express *tfoX* (Fig. 2). An isogenic strain was also constructed to serve as a control, T6SS^{-} defective killer, which also lacks the T6SS structural *vasK* gene (Fig. 1). Finally, a C6706 Δ*vasK* mutant that also lacks the

three immunity proteins (Δ*vasK* Δ*tsiV*1 Δ*tsiV*2 Δ*tsiV*3) serves as a model "prey" similar to *E. coli* bacteria (Fig. 1).

Fluorescence microscopy methods have been used in many prior studies to directly visualize the activity of T6SS$^+$ predator cells expressing Green Fluorescent Protein, GFP, against prey cells expressing Red Fluorescent Protein, RFP, and to verify that lysis of individual prey cells occurs only by direct predator-prey contact in experimental settings over a period of minutes (Fig. 3). These observations are consistent with 3 hour "killing assays" used in the Hammer lab and others showing that prey cell (ex: *E. coli*) numbers can be reduced by 5 orders of magnitude when grown on solid agar surfaces with "constitutive" *V. cholerae* T6SS$^+$ predator cells (Basler *et al.*, 2012; Watve, Thomas & Hammer, 2015). While the previous studies helped define the proximate mechanism for T6SS-mediated killing, it has not been quantified experimentally how spatial organization at the single cell level (degree of cell-cell contact) ultimately affects predator and prey on a population-wide scale.

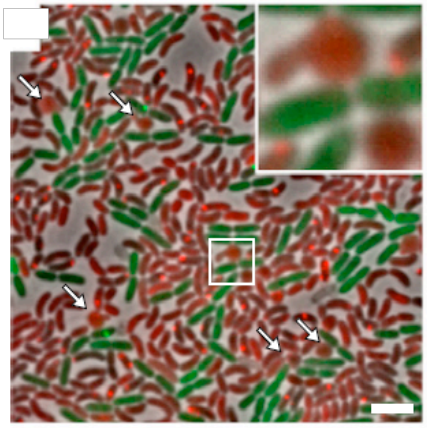

Figure. 3. Fluorescence microscopy with T6SS$^+$ predator cells expressing GFP and T6SS- prey cells expressing RFP. Fluorescence microscopy shows predator cells can kill prey cells e (lysed cells appear rounded) by direct cell-cell contact. (Basler *et al*., 2012)

Florescence microscopy has been used by other to determine the role of T6SS in cell-cell interactions, yet methods have not been developed to quantify the effects of T6SS-mediated killing in colonies mimicking mixed-species environmental biofilms. The

goals of this project were: 1) construct GFP and RFP expressing plasmids to be introduced by conjugation into *E. coli* and *V. cholerae* for florescence microscopy, and 2) perform pilot microbial spatial expansion assays with T6SS^{+} *V. cholerae* (predator tagged with GFP) and both T6SS^{-} *V. cholerae* and *E. coli* (prey tagged with RFP). This research predicted that T6SS-mediated killing would result in reorganization of the bacterial population in a growing colony that can be visualized by fluorescence microscopy.

CHAPTER 2

METHODS

Construction of the pUC-oriT-Ptac-GFP/RFP plasmids

To visualize populations of *V. cholerae* predator cells and prey cells by fluorescence microscopy required the construction of several plasmids to express fluorescent proteins (FP) in different cell types. I engineered two high copy plasmids, named pUC-oriT-Ptac-GFP (Fig. 4A) and pUC-oriT-Ptac-RFP (Fig. 4B), to express fluorescence proteins that can be visualized by fluorescence microscopy. Each plasmid has two major components, including the vector backbone and 3 DNA inserts: an origin of transfer (oriT), the Ptac promoter, and either the GFP or RFP gene, which all add additional features to the plasmid (Fig. 4). The vector, pUC18, is a high-copy plasmid with an origin of replication (ColE1) and ampicillin resistance (AmpR) gene. Because pUC18 cannot be transferred from *E. coli* into other cells without an oriT, the oriT insert allows the plasmids to be introduced from a donor (i.e: *E. coli*) into a recipient (i.e: *V. cholerae*) through

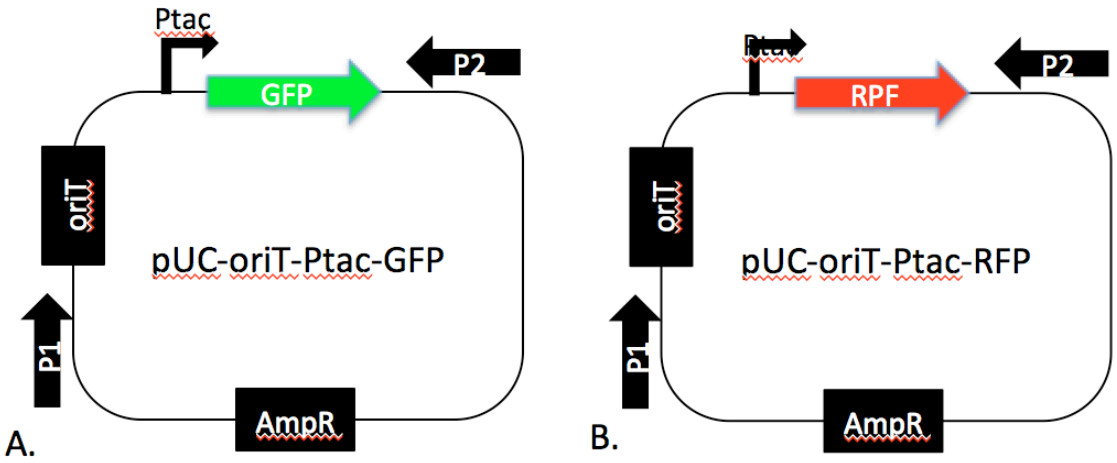

Figure. 4. Plasmid maps of pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP. pUC-oriT-Ptac-GFP plasmid (6A) and pUC-oriT-Ptac-RFP (6B) have the oriT, Ptac, and GFP/RFP fragments with Amp resistance gene. Two specific designed primers are shown as P1 and P2.

conjugation. In addition, the Ptac promoter was introduced into a position upstream of GFP and RFP gene to activate each constitutively.

To construct pUC-oriT-Ptac-GFP (Fig. 4A), pUC18 was digested with HindIII and XbaI digestion enzymes, and alkaline phosphatase was added to remove the phosphate group at the 5' end. These two actions prevent self-ligation of the plasmid. The template plasmids (pSLS3, pEVS143, and pJT389) for polymerase chain reaction (PCR) that have an oriT, the Ptac promoter, and the GFP gene were extracted from *E. coli* strain BH2766, BH1511, and JT389, respectively. The fragments of interest were amplified by PCR with specifically designed primers that have overlapping sequence (Fig. 5). The results of PCR were confirmed by gel electrophoresis, and the migration of each band was used to verify the size of each PCR product generated. Specifically flanking primers (see P1 and P2 in Fig. 4) were used to confirm that the constructed plasmids contained the ~1.6 kb insert.

The vector and the inserts were assembled into a plasmid through the Gibson reaction, which is an isothermal single-reaction method for assembling multiple overlapping DNA fragments (Gibson, 2009; Fig. 5). This reaction requires a 5' exonuclease, DNA polymerase, and DNA ligase (Gibson, 2009; Fig. 5). During the Gibson reaction, the exonuclease randomly removes nucleotides at the 5' end, creating single-stranded DNA overhangs (Gibson, 2009; Fig. 5). With similar corresponding sequences that anneal, DNA polymerase fills the gaps, and the ligase covalently joins the DNA of adjacent segments. In this experiment, three Gibson reactions were performed: 1) vector and water mixture (negative control), 2) vector, oriT, Ptac, and GFP mixture, 3) vector, oriT, Ptac, and RFP mixture. The negative control was used to test the pUC18 plasmids were fully digested. The second plasmid, pUC-oriT-Ptac-RFP (Fig. 4B), was constructed in a similar manner, except the RFP gene was amplified and engineered into the pUC18 plasmid rather than GFP gene.

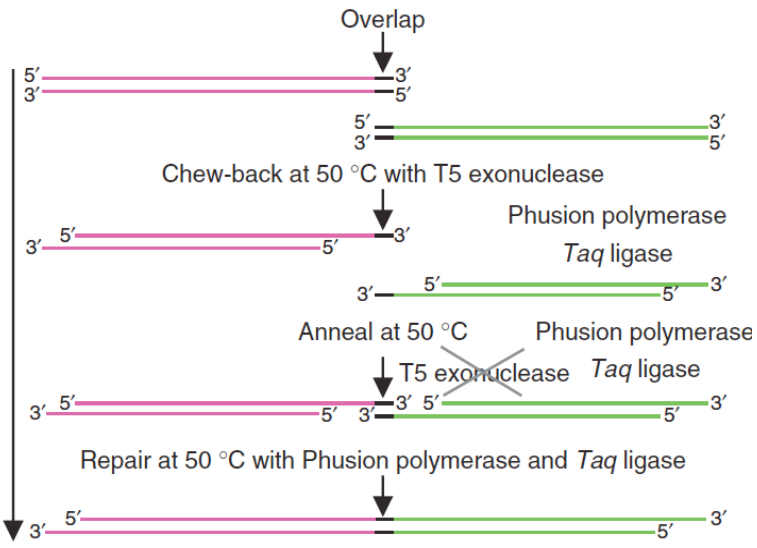

Figure. 5. Mechanism of Gibson Reaction *in vitro* DNA recombination (Gibson, 2009)

An aliquot of each Gibson reaction was introduced into an *E. coli* S17-1λpir diaminopimelic acid mutant (Dap^-) strain through electroporation. Because each plasmid has a ampicillin resistance gene, transformants were selected on LB agar medium

supplemented with Amp and Dap. Prior to transfer into *V. cholerae* predator and prey strains through conjugation, the plasmids were extracted and sequenced to confirm that the plasmids include all of the inserts, which were expected to be ~1.6kb long. Mating was performed to introduce pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP from *E. coli* into four *V. cholerae* strains: 1) wild type (WT) C6706; 2) the T6SS$^+$ "max killer" (constitutive TfoX strain); 3) the T6SS- defective killer (Δ*vasK* mutant) (Fig. 2); and 4) the triple immunity prey mutant (Δ*tsiV1,* Δ*tsiV2,* and Δ*tsiV3*; Fig. 1). However, the pUC-oriT-Ptac-GFP (Fig. 4A) and pUC-oriT-Ptac-RFP (Fig. 4B) plasmid were unstable in *V. cholerae,* and thus, they were not ready to be applied in fluorescence microscopy. Therefore, instead of using the plasmids I constructed, previously constructed *V. cholerae* strains, which have orange florescence protein (OFP) gene and cyan florescence protein (CFP) genes promoted by Ptac and integrated onto the chromosome, were used in florescence microscopy. These florescence proteins alleles had previously been shown to be stably expressed in *V. cholerae* when in single copy (Nadell, 2011), and have since been introduced into several predator and prey strains for on-going fluorescence microscopy experiments.

In this study, the *V. cholerae* C6706 T6SS$^+$ killer strain served as the predator, and the corresponding Δ*vasK* mutant strain served as a control. The prey was the *V. cholerae* triple immunity mutant. The predator strains carried the OFP gene in the chromosome, and the prey strain had the CFP in the chromosome.

Florescence Microscopy of Spatial Expansion

Each strain was incubated at 37°C for 24 hours in liquid LB medium, and then each overnight culture was diluted 1:10. Each diluted predator strain was combined with the diluted prey strain in a 1:1 and 1:10 ratio. The "max killer" strain was combined with triple immunity prey mutant. As a control, the Δ*vasK* mutant was combined with triple immunity mutant (negative control). A 0.5uL droplet of each mixed culture was placed on a thin LB layer on a microscope slide and incubated statically at 37°C overnight, followed by growth at room temperature for another 24 hours (Fig. 6). The colonies were visualized by florescence microscopy to observe the sectoring pattern and determine the distribution of predator and prey in the colony based on the color.

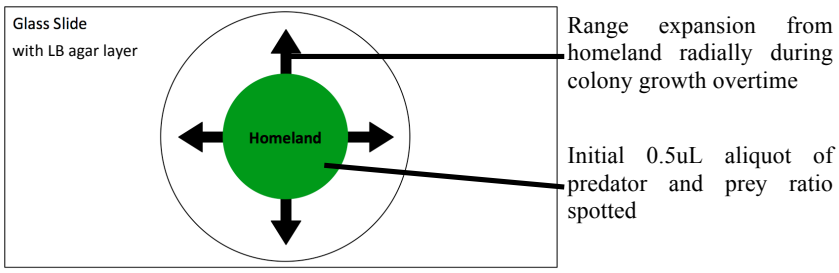

Figure. 6. A Model of Colony Range Expansion. A 0.5uL mixture of predator and prey culture was spotted on a glass slide with LB agar layer. Outer circle represents the predicted mature colony size. Green circle shows the homeland where the initial 0.5uL aliquot of predator and prey ratio spotted. Four arrows indicate the tread of range expansion from the homeland as the colony grows over 24-48 hours radially from center.

CHAPTER 3

RESULTS

Gel Electrophoresis of Cloned Sequences

To construct a plasmid that can encode GFP and RFP in *V. cholerae* required four inserts. Template plasmids for PCR were extracted from different *E. coli* strains (refer to method section). To get a sufficient amount of insert DNA from the plasmids, each insert was amplified by PCR and confirmed by gel electrophoresis. The appearance of the bands showed that each PCR was successful. Based on the migration of the bands, the size of the PCR product was determined. The size of oriT, Ptac, GFP, and RFP genes were expected to be 366bp, 165bp, 786bp, and 678bp, respectively. As shown in Fig. 7, the gel electrophoresis revealed that each PCR product appeared to be the correct size. The vector backbone was annealed with the inserts (oriT, Ptac, and GFP or RFP) through Gibson reaction to generate the pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP plasmids (Method section; Fig. 4).

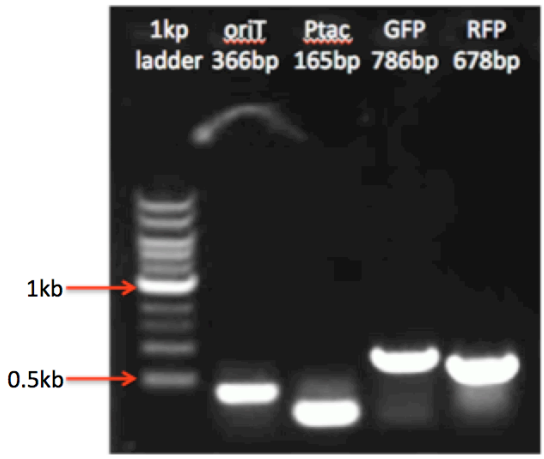

Figure. 7. Gel Electrophoresis Image of the Inserts. From left to right: 1kp ladder, oriT (366bp), Ptac (165bp), GFP (786bp),

To confirm that the candidate plasmids contained all of the inserts, the extracted plasmids from the transformants isolated on LB containing Amp and Dap were analyzed based on the phenotype, PCR, and Sanger sequencing results. From the *E. coli* Dap- strain, 11 pUC-oriT-Ptac-GFP candidates (1 of them turned green) and 5 pUC-oriT-Ptac-RFP candidates (1 of them turned red) were observed. Colony PCR was performed for each candidate with primers that annealed with the sequence upstream and downstream of the inserts (Fig. 1, Primer 1 and 2), and the PCR products were expected to be ~1600bp. Negative results were obtained from the first two trials (Fig. 8A and 8B). Although a

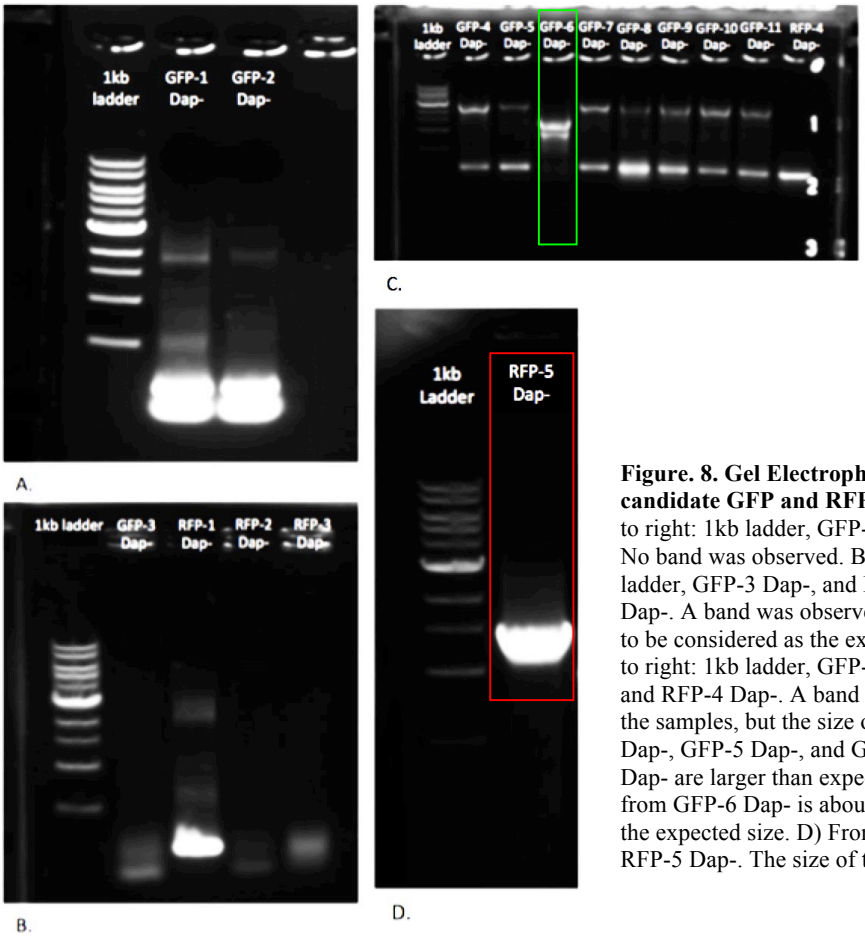

Figure. 8. Gel Electrophoresis Images of the candidate GFP and RFP plasmids. A) From left to right: 1kb ladder, GFP-1 Dap-, and GFP-2 Dap-. No band was observed. B) From left to right: 1kb ladder, GFP-3 Dap-, and RFP-1 Dap- to RFP-3 Dap-. A band was observed, but the size is too small to be considered as the expected result. C) From left to right: 1kb ladder, GFP-4 Dap- to GFP-11 Dap-, and RFP-4 Dap-. A band was observed from all of the samples, but the size of the band from GFP-4 Dap-, GFP-5 Dap-, and GFP-7 Dap- to GFP-11 Dap- are larger than expected. The size of the band from GFP-6 Dap- is about 1.5kb, which is closed to the expected size. D) From left to right: 1kb ladder, RFP-5 Dap-. The size of the band is about 1.5kb.

band was observed in Fig. 8B, the size was smaller than the expected product. A band was observed from GFP-4 Dap-, GFP-5 Dap-, and GFP-7 Dap- to GFP-11 Dap-, but the size is larger than 1.6kb (Fig. 8C). Two positive results, which were from the green

colonies (GFP-6 Dap-) and red colonies (RFP-5 Dap-), are shown in Fig. 8C (Green box) and 8D (Red box), and comparing to the 1kb ladder, the size of the bands is ~1500bp.

The plasmids from colonies that showed positive results by PCR were sent for sequencing to confirm that the expected inserts were in the pUC18 vector. The results showed that the plasmids from GFP-6 Dap- and RFP-7 Dap- had all of the expected inserts with no sequence errors. Based on the phenotype, PCR, and sequencing results, I concluded that GFP-6 Dap- and RFP-7 Dap- were correctly constructed for use in the experiment described below.

Analysis of Florescence Microscopy

Despite the correct sequence in each plasmid (pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP), *V. cholerae* strains carry the GFP and RFP expressing plasmids generated heterogeneous colonies that were both expressing the fluorescent protein, suggesting the plasmids I constructed were stressful to *V. cholerae* and resulted in viability loss (data not shown). Because we had experienced prior similar stability issues with GFP-expressing plasmids, the decision was made to not use *V. cholerae* strains carrying GFP- and RFP-expressing plasmids (Liu et al., 1999). Alternatively, Orange Fluorescent Protein (OFP) and Cyan Fluorescent Protein (CFP) alleles constructed by Jacob Thomas, a post-doc in the Hammer lab, were introduced in single copy onto the chromosome of *V. cholerae* predator and prey strains for use in fluorescence microscopy. These alleles were stable in single copy and did not generate white colonies on plates. These strains were used in the range expansion assays.

The *V. cholerae* OFP-expressing predator strains (The TfoX* "max killer" and the Δ*vasK* negative control) and *V. cholerae* CFP-expressing prey strain (triple immunity mutant that lacks immunity to T6SS-mediated killing) were mixed at 1:1 and 1:10 ratios (Fig. 6), incubated, and view by fluorescence microscopy. Preliminary images documented the spatial expansion of colonies as shown in Fig. 9.

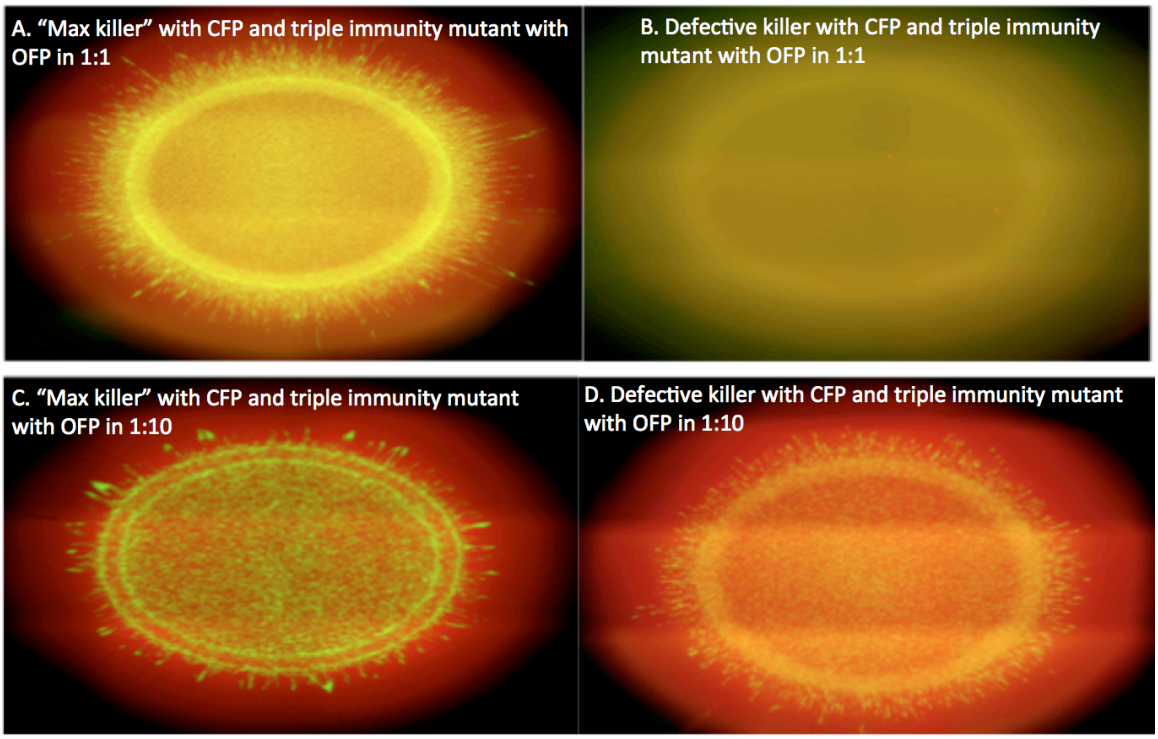

Figure. 9. Pictures of colonies with mixed *V. cholerae* strains. A) Mixed "max killer" with CFP (Green) and triple immunity mutant with OFP (Red) in 1:1 ratio. B) Mixed Δ*vasK* mutant with CFP (Green) and triple immunity mutant with OFP (Red) in 1:1 ratio. C) Mixed "max killer" with CFP (Green) and triple immunity mutant with OFP (Red) in 1:10 ratio. D) Mixed Δ*vasK* mutant with CFP (Green) and triple immunity mutant with OFP (Red) in 1:10 ratio.

The preliminary results surprisingly showed the OFP prey population (red) was dominant in all of the colonies except the one shown in Fig. 9B. Moreover, a bright circle was observed in all of the colonies, and sectoring was observed in the outer region of the colony where the expansion occurred. In the 1:1 ratio of *V. cholerae* constitutive TfoX "max killer" and triple immunity mutant strains, the green and red was equally mixed on and inside the circle, which showed bright yellow color (Fig. 9A). Red from the prey

covered most of the space outside out the circle as well as many yellow, thin, and sharp sectors (Fig. 9A). As the negative control for the 1:1 ratio, which the Δ*vasK* mutant was the predator while the prey remained the same, there were no red cells observed (Fig. 9B). In the 1:10 ratio of the same combination, instead of yellow, green color was observed in and inside the circle, and outside the circle, the red color was still dominant with some green sectoring (Fig. 9C). Comparing to the negative control for the 1:10 ratio, red color was observed to be dominant in the negative control because no killing event occurred which resulted an orange circle and sectors, but the color outside of the circle remained the same (Fig. 9D). We hypothesized that the overexpression of TfoX in the "max killer" may impose a growth defect on this strain in this long-term experiment. As a result, allelic exchange was performed to introduce the OFP allele into a *V. cholerae* strain that were engineered to be constitutive for QstR* rather than TfoX* (Fig. 1). This strain, the "killer" was used by me and others in the lab in subsequent experiments as constitutive QstR expression did not impose a burden on *V. cholerae*.

Using the OFP-expressing constitutive QstR "killer" strain and isogenic Δ*vasK* mutant as predators against a CFP-expressing triple immunity mutant, we observed that an active T6SS in the *V. cholerae* predator (red) completely eliminated prey (green) from the homeland (Fig. 6), despite an initial ratio of 1 predator:10 prey (Fig. 10, left). We

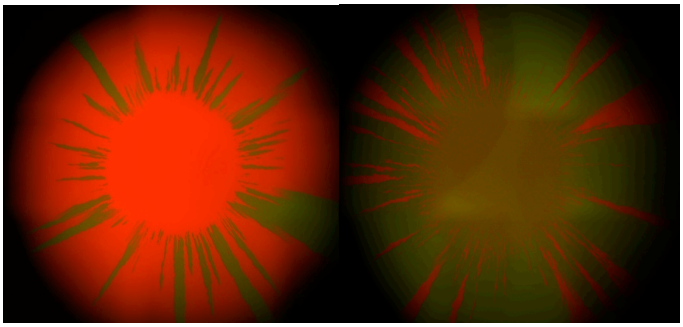

Figure. 10. T6SS-mediated killing alters distribution of prey in a range expansion. Left: *V. cholerae* C6706 T6SS^{+} "Killer" (constitutive QstR) versus *V. cholerae* triple immunity prey mutant (constitutive QstR, *ΔvasK*, triple immunity genes deleted) in 1:10 ratio.
Right: *V. cholerae* C6706 T6SS^{-} "Defective Killer" (constitutive QstR, Δ*vasK*) versus *V. cholerae* triple immunity prey mutant (constitutive QstR, *ΔvasK*, triple immunity genes deleted) in 1:10 ratio.

note that some prey survive in the area of the range expansion. In contrast, the defective killer was unable to kill prey, resulting in domination of prey (green) in the homeland and range expansion (Fig. 10, right) at the same initial ratio of 1 predator:10 prey. Thus, T6SS-mediated killing altered the spatial distribution of the prey in this simple range expansion assay.

CHAPTER 4

DISCUSSION

Fluorescence microscope has been used to study and visualize *V. cholerae* T6SS activities in cell-cell interaction since 2012 (Basler *et al.*, 2012). However, the effect of T6SS in a complex heterogeneous community (ex: a mixed-species colony) remains unclear. The focus of this study was 1) to construct *V. cholerae* strains carrying distinct fluorescent proteins and then 2) to visualize the organization of *V. cholerae* predator and prey in a spatial expansion model, where each strain expresses either fluorescent protein. The bacterial population structure was expected to be changed due to the killing events from T6SS in *V. cholerae*. As the colony expends, the T6SS$^+$ "killer" predator was predicted to dominate. In contrast, we predicted that in a colony carrying a predator defective in killing (the Δ*vasK* mutant), the ratio of two populations should be unaltered as the colony expands.

The newly engineered plasmids, pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP (Fig. 4), were designed to be used throughout this study, and the aim of the plasmids is to introduce into the predators and preys, so they will be able to express GFP and RFP constitutively for identification under fluorescence microscope. However, although both plasmids were successfully constructed and transferred into *V. cholerae*, the plasmid seemed unstable in *V. cholerae*, and colonies often arose that appeared to no longer express the fluorescence. Some of the colonies no longer appeared to express the fluorescent proteins, and others also failed to grow upon restreak (Data not shown). We concluded that the multi-copy plasmids expressing GFP and RFP might be having a

negative effect on growth (Liu et al., 1999). Therefore, previously constructed OFP and CFP expressing strains were used instead of the pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP plasmids (Fig. 4).

Range expansion has been used for analysis of colony development in microbiology (Hallatschek *et al.*, 2007; Müller *et al.,* 2014). To analyze the organization of two populations in a mixed colony on solid agar, fluorescence microscopy was performed with the strains that expressed OFP and CFP, and colonies observed by fluorescence microscopy (Fig. 9). Our results showed that the cells were able to express OFP and CFP on solid LB agar. The lack of red cells in Fig. 9C, may represent complete killing of the prey which could be an experimental error that the strain was not added to the culture. Although the fluorescence proteins are supposed to be orange and cyan, the picture showed red and green due to the limit of the filter in the fluorescence microscope, which only has red and green filters available. All of the pictures showed an inner bright circle where the initial aliquot was plated, and growth outside of the circle developed by expansion of the colony by "pioneers". As the cells in the colony grow and divide, the first cell "pioneers" grow outside of the circle and take advantage of nutrients there. Because T6SS$^+$ predators should compete effectively with prey when initially plated together in the center of the colony, we expected to see more or larger sectors of the CFP-labeled predator.

In the mixture of *V. cholerae* constitutive TfoX "max killer" and the triple immunity mutant, the max killer was expected to dominate in the colony due to the

killing events. In contrast, an equal distribution of predator and prey was expected in the mixture of the T6SS$^-$deficient Δ*vasK* mutant and the triple immunity mutant because the Δ*vasK* mutant is a defective killer. However, based on the results, the red color, which was produced by the prey, was dominant in all of the colonies with the exception of the "max killer" and triple immunity mutant in 1:10 ratio combination (Fig. 9C). In addition, the sectoring was not obvious as the area outside of the circle was covered with red from the prey cells and the green sector was thin, short, and sharp (Fig. 9). The result was not expected because the prey appeared to be more successful than T6SS$^+$ constitutive TfoX predator and dominate at the edge of the growing colony.

When grown alone on solid LB agar plates, we recently observed that the "max killer" predator cells expressing the T6SS constitutively form smaller colonies than the triple immunity prey cells that are Δ*vasK* and express no T6SS apparatus (data not shown). We reasoned that perhaps these growth differences also affect the competition of the predator and prey in the colony expansion assay, allowing the prey to be more successful than expected. As a result, we recently constructed predator and prey more carefully. *V. cholerae* strains are expressing *qstR* and not tfoX constitutively, to rule out contributions of and grown rate differences.

Although the results with the plasmids I constructed were not expected and likely the result of growth defects, this study provided useful information to initiate design and improve the future experiments. For example, we have made progress determining appropriate fluorescent proteins to express in *V. cholerae*, and determined it is more

suitable to express single copy fluorescence alleles from the chromosome and not from a very high copy plasmid like pUC (Fig. 4). Although *V. cholerae* can carry pUC-oriT-Ptac-GFP and pUC-oriT-Ptac-RFP, there appears to be a burden associated with carrying these multi-copy plasmids (Fig. 4). It is possible that we may still test the utility of lower copy plasmids carrying OFP and CFP to explore T6SS-mediated killing using a set of environmental isolates of *V. cholerae* that do not appear to be amenable to integration of the fluorescent alleles on the chromosome.

Moving forward, I am now initiating new range expansion experiments competing the C6706 constitutive QstR T6SS$^+$ "killer" strain (carrying chromosomal OFP) with a T6SS$^+$ environmental *V. cholerae* competitor identified by others in the Hammer lab to be capable of T6SS-mediated killing of both *E. coli* and *V. cholerae* C6706 (data not shown). These "mutual killers" are now being piloted in an experimental evolution protocol I am beginning to evolve the C6706 strain into a more effective competitor by repeat passage in range expansions with the environmental isolate. These new studies were made possible by the pilot experiments described here. This new direction into experimental evolution may help to uncover additional factors that play a role in T6SS$^-$ mediated competition.

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